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Report

p53-Dependent Translational Control of Senescence and Transformation via 4E-BPs

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SUMMARY

eIF4E, the mRNA 5′ cap-binding translation initiation factor, is overexpressed in numerous cancers and is implicated in mechanisms underlying oncogenesis and senescence. 4E-BPs (eIF4E-binding proteins) inhibit eIF4E activity, and thereby act as suppressors of eIF4E-dependent pathways. Here, we show that tumorigenesis is increased in p53 knockout mice that lack 4E-BP1 and 4E-BP2. However, primary fibroblasts lacking 4E-BPs, but expressing p53, undergo premature senescence and resist oncogene-driven transformation. Thus, the p53 status governs 4E-BP-dependent senescence and transformation. Intriguingly, the 4E-BPs engage in senescence via translational control of the p53-stabilizing protein, Gas2. Our data demonstrate a role for 4E-BPs in senescence and tumorigenesis and highlight a p53-mediated mechanism of senescence through a 4E-BP-dependent pathway.

INTRODUCTION

Translation initiation is deregulated during tumorigenesis (Schneider and Sonenberg, 2007). The translation initiation factor, eIF4E, is an oncogene that regulates the translation of a specific subset of tumor-promoting mRNAs (Graff et al., 2007; Mamane et al., 2007; Schneider and Sonenberg, 2007). eIF4E, like other oncogenes (e.g., Akt or Ras) induces senescence, which acts as an intrinsic barrier to cancer (Campisi and d'Adda di Fagagna, 2007; Collado et al., 2007; Lowe et al., 2004). Loss of tumor suppressors (e.g., p53 and $p19^{ARF}$) impairs senescence and promotes cell transformation (Miyauchi et al., 2004; Nogueira et al., 2008; Serrano et al., 1997). Because eIF4E activity is negatively regulated by the eIF4E-binding proteins (4E-BPs), we reasoned that eIF4E-induced senescence may be regulated by 4E-BPs. Here, we describe the translational control of senescence and transformation by 4E-BPs.

RESULTS

To assess the role of 4E-BPs in tumorigenesis in vivo, we compared tumor-free survival of $p53^{-/-}$ with $4E\text{-}BP1^{-/-}$ $4E\text{-}BP2^{-/-}$ $p53^{-/-}$ triple knockout (TKO) mice. As shown previously, $p53^{-/-}$ mice exhibited a high incidence of tumors, especially lymphomas (Donehower et al., 1992), with a mean tumor-free survival time of 23 weeks. TKO mice developed similar types of tumors, but the tumor-free survival was reduced (15 versus 25 weeks; $p < 0.001$), as compared with $p53^{-/-}$ mice (Figure 1A). Interestingly, the survival of $p53^{+/-}$ mice was not reduced by the absence of 4E-BPs (see Figure S1 available online), suggesting

SIGNIFICANCE

Mounting evidence shows that eIF4E is an important oncogene, which is currently a target for anticancer drugs in clinical trials. Poor prognosis correlates with increased eIF4E expression levels and increased phosphorylation of its major cellular inhibitor, 4E-BP1. Cells lacking 4E-BPs undergo p53-dependent senescence and are resistant to oncogenic transformation. This phenomenon is explained by the translational control of Gas2 expression. These genetic data demonstrate that targeted disruption of 4E-BP expression causes elevated expression of the p53-tumor suppressor protein. Our findings are significant for anticancer drug development because they show that the p53 status must be considered in studies of therapeutic targeting of the translation initiation pathway.

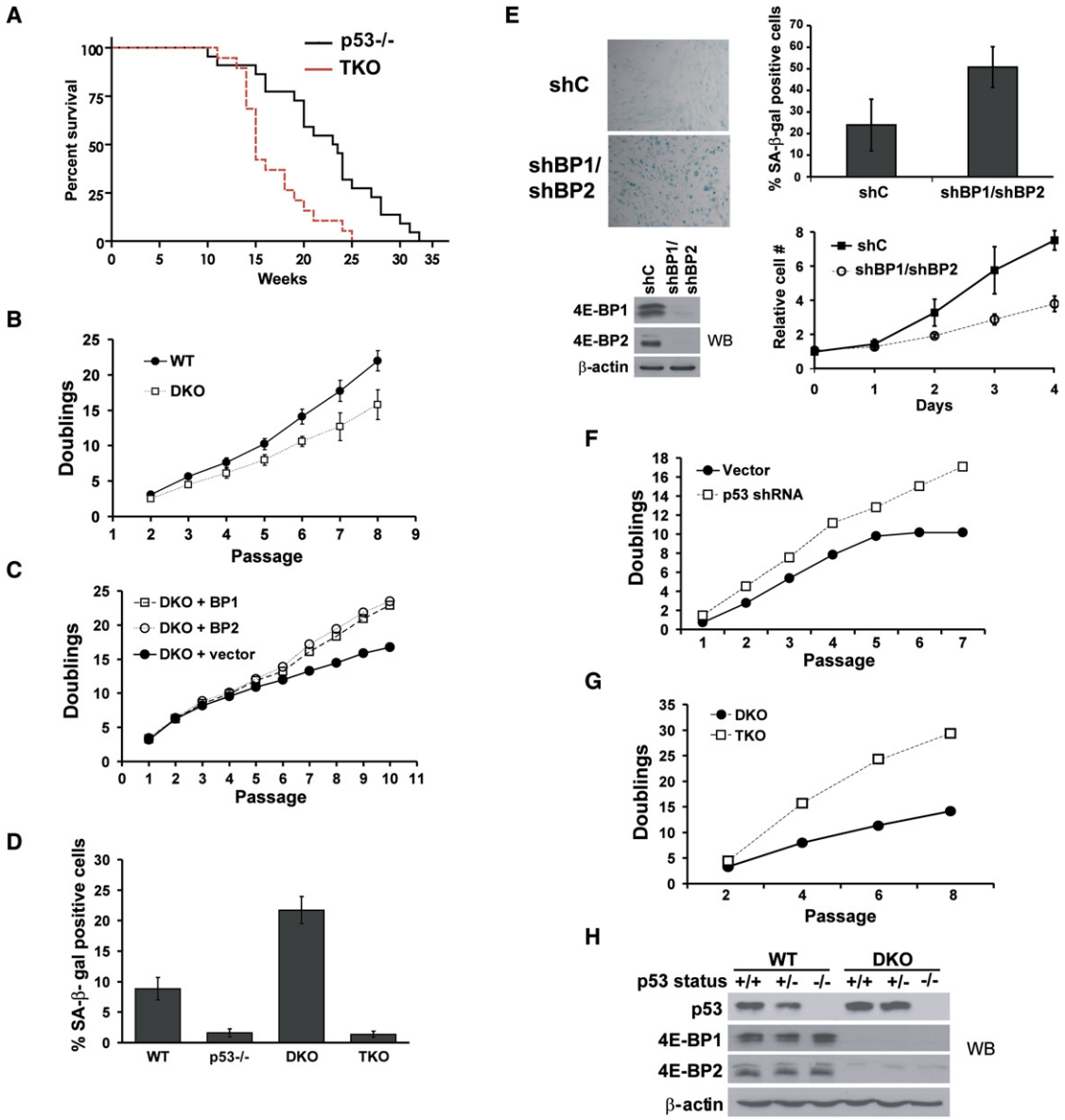

Figure 1. 4E BPs Control p53 Dependent Senescence

(A) Kaplan Meier plot showing survival of *p53* $^{/}$ and *4E BP1* $^{/}$ *4E BP2* $^{/}$ *p53* $^{/}$ triple knockout (TKO) mice. Median survival: *p53* $^{/}$ = 23 weeks (n = 19), TKO = 15 weeks (n = 19); $p < 0.001$.

(B) Population doublings (PDs) for WT and DKO MEFs were determined for eight passages. MEFs from three independent pairs of embryos were examined. Error bars indicate the standard deviation (SD).

(C) PDs were determined for DKO MEFs transduced with MSCV IRES GFP (vector), MSCV 4E BP1 IRES GFP, or MSCV 4E BP2 IRES GFP (n = 3).

(D) SA β gal positive cells (%) in WT, *p53* $^{/}$, DKO, and TKO MEF cultures at passages 4, 5, and 6. The average is shown for each genotype. Error bars indicate the SD (n = 3).

(E) MRC5 cells infected with control lentivirus (shC) or with sh4E BP1 and sh4E BP2 lentiviruses together (shBP1/shBP2). SA β gal staining of MRC5 cells (day 4; upper left) and western blots (lower left) showing 4E BP1 and 4E BP2 silencing. Quantification of % SA β gal positive cells (day 2; upper graph) and proliferation (0 to 4 days; lower graph) are indicated in the graphs showing averages. Error bars represent SD (n = 3). WB, western blot.

(F) PDs for DKO MEFs transduced with p53shRNA or MSCV IRES GFP retroviruses (n = 3).

(G) PDs for DKO and TKO MEFs were determined for 8 passages. The values are an average of two independent experiments.

(H) Western blot analysis of p53, 4E BP1, and 4E BP2 expression levels in WT and DKO MEFs with varying p53 status (+/+, +/ , /). β actin served as a loading control. WB, western blot.

that a role for 4E-BP in tumorigenesis varies depending on the amount of p53 in the cell. These results demonstrate that in the complete absence of p53, 4E-BPs increase tumor-free survival.

We further studied the role of 4E-BPs in tumorigenesis using primary murine embryonic fibroblasts (MEFs). The population doubling rate of *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ double knockout (DKO)

MEFs was reduced by 37% as compared with wild-type (WT) MEFs. At passage 8, WT MEFs had undergone 22 ± 3 population doublings (PDs), relative to 16 ± 2 PDs for DKO MEFs (Figure 1B). Between passages 4 and 8, DKO MEFs also exhibited a > 50% reduction in the number of cells in S phase, as compared with WT MEFs (data not shown). To confirm the role of 4E-BPs in the proliferative defect of DKO MEFs, 4E-BP1 or 4E-BP2 was re-expressed in DKO MEFs using retroviral vectors. Expression of 4E-BP1 or 4E-BP2 in DKO MEFs engendered faster growth beyond passage 6 (Figure 1C). These data demonstrate that the slow growth of DKO MEFs is due to the lack of 4E-BPs.

Reduced growth of DKO MEFs could be caused by increased cell death or senescence. Increased apoptosis was not observed (data not shown). However, DKO MEFs exhibited a 2.5–fold increase in the number of cells staining positive for senescence-associated β-galactosidase (SA-β-gal) activity, as compared with WT MEFs (21.7% ± 2.2% versus 8.8% ± 1.8%, $p < 0.05$) (Figure 1D). This finding is not restricted to MEFs, because shRNA silencing of *4E-BP1* and *4E-BP2* in MRC-5 human lung fibroblasts caused morphological flattening, a 50% reduction in cell proliferation rate, and a 2-fold increase in senescent cells (Figure 1E). Thus, the lack of 4E-BPs impedes cell proliferation due to premature senescence. eIF4E overexpression in MEFs also induces senescence (Ruggero et al., 2004), suggesting that these findings are caused by increased eIF4E activity due to the absence of 4E-BPs.

Because p53 is a central regulator of senescence (Serrano et al., 1997), we investigated whether increased senescence in DKO MEFs was p53 dependent. Less than 2% of $p53^{-/-}$ or TKO MEFs (1.6% and 1.4%, respectively) exhibited SA-β-gal activity (Figure 1D), demonstrating that p53 is required for DKO MEFs to undergo senescence. For corroboration of these findings, p53 was silenced in DKO MEFs, which reversed the growth phenotype of DKO MEFs (Figure 1F). Furthermore, TKO MEFs underwent many more PDs than DKO MEFs after eight passages (Figure 1G; 29 PDs versus 13 PDs, respectively). Finally, p53 protein levels were increased by 1.5- and 2-fold in DKO MEFs ($p53^{+/+}$ and $p53^{+/-}$, respectively; passage 6), as compared with $p53^{+/+}$ and $p53^{+/-}$ MEFs, respectively (Figure 1H), demonstrating that senescence correlates with increased p53 expression. Thus, DKO MEFs express elevated p53 protein levels and undergo p53-dependent senescence.

p53-mediated senescence antagonizes tumorigenesis (Chen et al., 2005; Deng et al., 2008; Lowe et al., 2004). To examine whether the increase in p53-dependent senescence observed in DKO MEFs renders these cells resistant to transformation, MEFs were infected with a retrovirus expressing both E1A and Ras proteins. E1A/Ras-expression in WT MEFs caused 2.5 ± 0.3 more focus formation than in DKO MEFs (Figure 2A). The empty retrovirus or that expressing E1A alone failed to transform cells (Figure 2A). Introduction of 4E-BP1 or 4E-BP2 into DKO MEFs caused more than a 2-fold increase in the number of foci formed when E1A and Ras were overexpressed (Figure S2). Furthermore, E1A/Ras-expressing WT MEFs formed subcutaneous tumors in nude mice, whereas E1A/Ras-expressing DKO MEFs failed to do so (Figure 2B). To determine whether p53 expression levels modulated transformation, we compared the effect of E1A/Ras in WT and DKO MEFs harboring varying amounts of p53 protein by using $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ MEFs. Similar to the E1A/Ras-induced transformation rates in WT and DKO MEFs, DKO ($p53^{+/-}$) MEFs formed 1.9- ± 0.4-fold fewer foci as compared with $p53^{+/-}$ MEFs (Figure 2C). In contrast, both $p53^{-/-}$ and TKO MEFs formed more than ten times the number of foci observed in $p53^{+/+}$ and $p53^{+/-}$ MEFs (Figure 2C). Consistent with the focus formation assays, p53-expressing DKO MEFs ($p53^{+/+}$ or $p53^{+/-}$) also formed ~3-fold fewer colonies in soft agar, as compared with WT MEFs ($p53^{+/+}$ or $p53^{+/-}$ MEFs) (Figure 2D). The reduction in the number of colonies formed by DKO MEFs was not seen in the absence of p53 (Figure 2D). Retroviral transduction efficiency, as monitored by Ras protein expression, was similar in all the MEFs examined (Figure 2E). These results show that the transformation of $4E\text{-}BP1^{-/-}4E\text{-}BP2^{-/-}$ DKO MEFs is impaired by the p53 pathway and loss of 4E-BPs enhances transformation in $p53^{-/-}$ cells.

Given the increase in p53 protein expression in DKO MEFs, we wished to elucidate the molecular mechanism of p53 regulation. p53 mRNA levels in primary WT and DKO MEFs were not different (Figure S3). Furthermore, p53 mRNA translation rates were similar in WT and DKO MEFs (see below and Figure 3D), suggesting that p53 protein expression was controlled at a post-translational level in DKO MEFs. Indeed, p53 protein stability was increased in both primary and E1A/Ras expressing DKO MEFs, as compared with WT MEFs (Figures S4A and S4B, respectively). Neither $p19^{ARF}$ nor p16 protein levels increased in DKO MEFs (Figure S4A), suggesting that p53 was stabilized independently of changes in the $p19^{ARF}$/Mdm2 pathway (Pomerantz et al., 1998).

p53 can also be stabilized through the Gas2 protein, which inhibits m-calpain, a calcium-dependent protease (Benetti et al., 2001). Gas2 mRNA and protein levels increase upon serum withdrawal and diminish by serum stimulation (Benetti et al., 2005; Schneider et al., 1988). To determine whether Gas2 protein levels are regulated by the 4E-BPs, we grew MEFs in low serum (0.1%) for 24 hr and then stimulated them with 20% serum. After 48 hr of stimulation, Gas2 protein levels decreased by 75% in WT MEFs, as compared with unstimulated cells (Figure 3A; comparing 0 and 48 hr). In contrast, DKO MEFs maintained a high level of Gas2 protein, even after 48 hr of serum stimulation when the level of Gas2 protein was approximately 5-fold higher, as compared with WT MEFs (Figure 3A). Serum stimulation also caused an increase in p53 protein expression, in agreement with earlier results (Reich and Levine, 1984). Consistently, maintenance of high Gas2 protein levels upon serum stimulation correlated well with elevated p53 protein levels (>3-fold by 24 hr; Figure 3A). Gas2 mRNA levels decreased to a similar extent (90% and 80%) in WT and DKO MEFs, respectively (Figure S5A). Thus, Gas2 protein levels are elevated in DKO MEFs primarily through a posttranscriptional process. The regulation of Gas2 was also examined in $p53^{-/-}$ and TKO MEFs. Although serum stimulation caused a 90% decrease in Gas2 mRNA levels in both $p53^{-/-}$ and TKO MEFs (Figure S5B), TKO MEFs expressed > 2-fold more Gas2 protein, as compared with $p53^{-/-}$ MEFs after 48 hr of stimulation (Figure 3B). Consistent with the above data, actinomycin D treatment caused a similar reduction in Gas2 mRNA levels in 4E-BP null and control MEFs (Figure S6). Elevation of Gas2 protein in DKO MEFs is

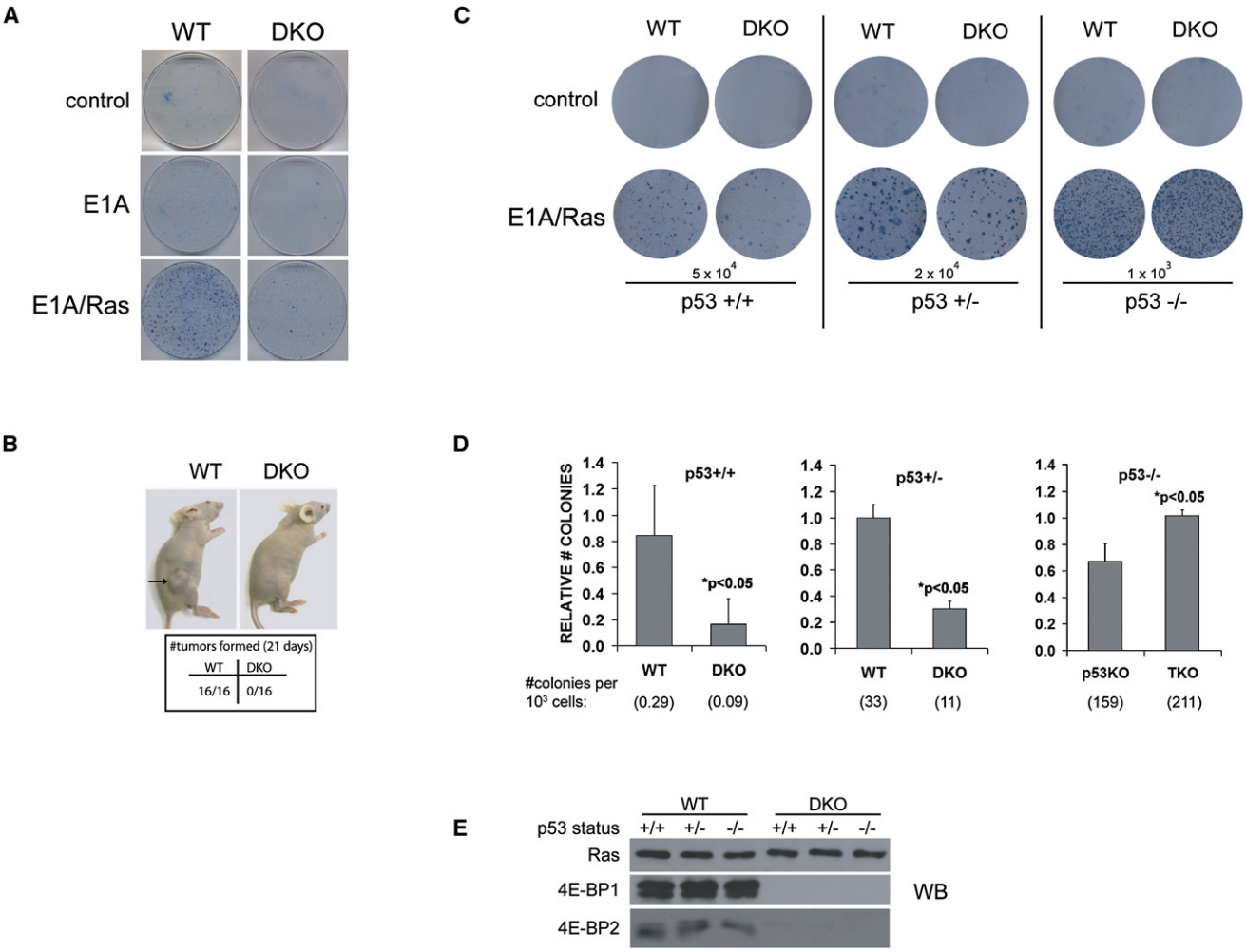

Figure 2. Loss of 4E BP Expression Renders MEFs Resistant to E1A/Ras Induced Transformation in a p53 Dependent Manner

(A) Focus formation assay showing WT and DKO MEFs at 2 weeks after infection with retroviruses (pLPC [empty control vector], E1A, or E1A/Ras) (n = 4).

(B) Tumor growth of E1A/Ras expressing WT (left, arrow) and DKO (right) MEFs in nude mice. Each cell type was injected into 16 mice and growth was monitored for 3 weeks.

(C) Focus formation assay showing the effect of E1A/Ras overexpression in WT, DKO, $p53^{+/}$, DKO ($p53^{+/}$), $p53^{/}$, and TKO MEFs. Cells were transduced with E1A/Ras or vector control. The number of cells seeded (depending on the p53 status) is indicated (n = 3).

(D) Growth of E1A/Ras expressing MEFs from (C) in soft agar. The graphs indicate the differences in the number of colonies observed from WT to DKO MEFs with varying p53 status ($p53^{+/+}$ [left], $p53^{+/}$ [middle], or $p53^{/}$ [right]). The number of colonies formed per 10^3 cells is indicated below each graph. Error bars indicate SD (n = 3; independent sets of primary MEFs).

(E) Western blot analysis showing Ras levels in transformed MEFs used in (C) and (D). WB, western blot.

expected to reduce the protease activity of m-calpain, because Gas2 binding to m-calpain impairs its activity (Benetti et al., 2001). Indeed, lower calpain activity (49% ± 7%, $p < 0.05$) was detected in DKO MEF extracts (Figure 3C, upper panel). Reduced calpain activity in DKO MEFs (51% ± 12%, $p < 0.05$) was also detected in vivo using flow cytometry to monitor the cleavage of the calpain-specific substrate, t-BOC-LM-CMAC (Figure 3C, lower panel). Taken together, these data demonstrate that Gas2 protein expression is controlled by 4E-BPs at the posttranscriptional level.

Because 4E-BPs are translational regulators (Pause et al., 1994), we assessed the translational efficiency of Gas2 mRNA in 4E-BP DKO MEFs using sucrose gradients to partition efficiently translated mRNAs (enriched on polysomes) from poorly translated mRNAs. Heavy sedimenting polysome fractions from DKO MEFs contained 1.94 ± 0.5 ($p < 0.05$) more Gas2 mRNA than WT MEFs (Figure 3D). In contrast, the polysome distribution of p53 and $p19^{ARF}$ mRNAs was not altered in DKO MEFs (Figure 3D). Thus, the elevated levels of Gas2 protein in DKO MEFs are caused by a selective increase in translational initiation of Gas2 mRNA, most likely mediated by an increase in eIF4E activity because of the absence of 4E-BPs. Consistent with this notion, eIF4E overexpression caused an increase in Gas2 protein (Figure S7). Taken together, these data demonstrate that DKO MEFs express more Gas2 protein as a result of an increase in Gas2 mRNA translation initiation.

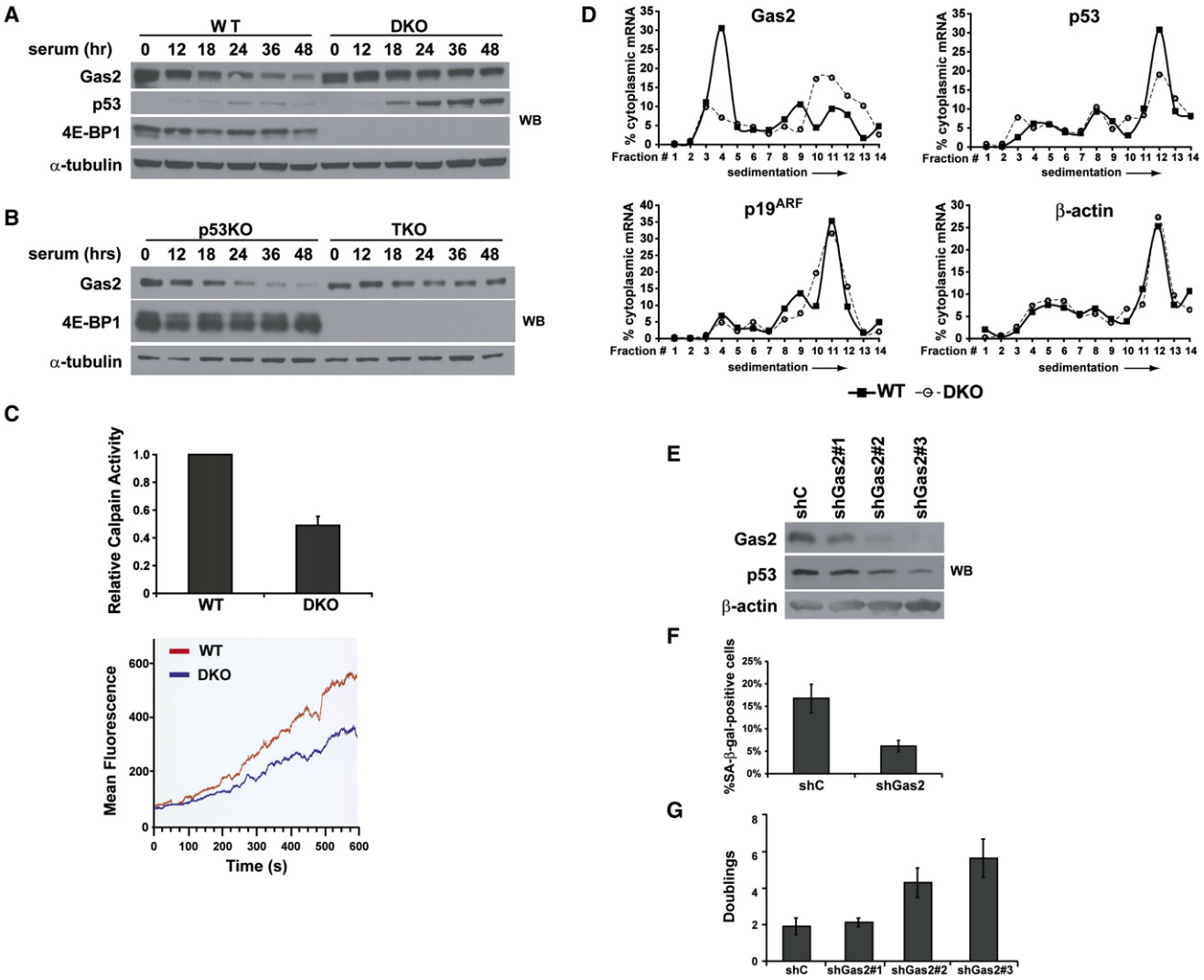

Figure 3. Increased Gas2 mRNA Translation Causes Senescence in DKO MEFs

(A) Western blot analysis of p53, Gas2, and 4E BP1 levels in WT and DKO MEFs after serum stimulation. WB, western blot.

(B) Western blot analysis of Gas2 and 4E BP1 protein levels in *p53* $^{-/-}$ and TKO MEFs after serum stimulation. α Tubulin served as a loading control in (A) and (B) (results are representative of two independent experiments). WB, western blot.

(C) Shown in the upper panel is a graph representing the relative calpain activity in independent primary WT and DKO MEF extracts. Error bars indicate the SD (n = 3). Shown in the lower panel is mean fluorescence representing calpain activity in WT (red) and DKO (blue) MEFs; the cleavage product was continuously measured for 570 s (n = 3).

(D) Graphs showing the distribution of Gas2, p53, p19^{ARF}, and β actin mRNA in sucrose gradients prepared from MEFs after 24 hr of serum stimulation. The distribution of each mRNA, as determined using RT qPCR, is shown for WT (closed squares, ■) and DKO MEFs (open circles, ○).

(E) Western blot showing expression of Gas2 protein in DKO MEFs postsilencing by Gas2 shRNAs (shGas2#1, shGas2#2, shGas2#3) and control, shC. β actin served as a loading control. WB, western blot.

(F) Graph showing % SA β gal positive cells in control cells (shC) or following silencing of Gas2 (shGas2#2 or shGas2#3) in DKO MEFs. Error bars indicate the SD (n = 3).

(G) Population doublings (after four passages) for DKO MEFs transduced with shGas2#1, shGas2#2, shGas2#3, or shC.

Gas2 is implicated in p53-induced growth inhibition (Kondo et al., 2008). Given the negative regulation of Gas2 translation by 4E-BPs, we asked whether Gas2 is necessary for the premature senescence in DKO MEFs. To this end, Gas2 expression was silenced in DKO MEFs (passage 3) using Gas2-specific shRNAs or a nonsilencing shRNA, shC. The varying degrees of Gas2 reduction caused by the different shRNAs were paralleled by similar reductions in p53 protein expression (Figure 3E). After two passages, 17% of DKO MEFs expressing shC exhibited SA-β-gal activity (Figure 3F). However, SA-β-gal activity was reduced to 6% when Gas2 was silenced in DKO MEFs (Figure 3F). Furthermore, Gas2-specific shRNAs caused an increase in cell proliferation (2.1 ± 0.2, 4.3 ± 0.8, and 5.6 ± 1.0 PDs, $p < 0.05$, for the different Gas2 shRNAs) (Figure 3G). DKO MEFs expressing shC shRNA underwent 1.9 ± 0.5 PDs (Figure 3G). Importantly, each Gas2 shRNA promoted growth

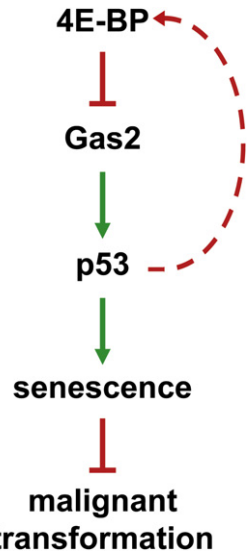

Figure 4. Model Showing the Control of p53 Dependent Senescence by 4E BPs

4E BPs inhibit translation of Gas2 mRNA whose product stabilizes p53 by binding m calpain and inhibiting its protease activity. p53 induces senescence and resistance to transformation. Induction of p53 may also cause transla tional inhibition through 4E BP activation as a negative feedback mechanism (Constantinou and Clemens, 2005, 2007). In the absence of 4E BPs, this nega tive feedback mechanism is impaired.

enhancement in proportion to the extent of Gas2 silencing. These data demonstrate that 4E-BPs control p53-dependent senescence by regulating Gas2 translation.

DISCUSSION

Aberrant translational control is a common feature in cancer (Schneider and Sonenberg, 2007). Accordingly, eIF4E and its 4E-BP suppressors play an important role in malignant transformation. In this study we unveiled a mechanism of translational control in cancer, which demonstrates a role for 4E-BPs in p53-dependent tumorigenesis. Akin to 4E-BPs, tumor suppressors, such as E2F, may exhibit context-dependent roles in tumorigenesis (Yamasaki et al., 1998). We show that 4E-BPs suppress p53-dependent senescence through the translational repression of Gas2 mRNA (see model, Figure 4). Conversely, in cells lacking p53, 4E-BPs inhibit neoplastic growth. Because cells that lack 4E-BP expression have higher levels of Gas2 protein and undergo p53-dependent senescence, our findings are consistent with a recent report demonstrating the requirement of p53 for Gas2-mediated growth inhibition (Kondo et al., 2008).

Our finding that deletion of 4E-BPs in p53 knockout mice reduces tumor-free survival may have important implications for human disease. It was reported that lower amounts of 4E-BP1 in childhood rhabdomyosarcoma (Petricoin et al., 2007) or its inactivation through hyperphosphorylation in several cancers, including ovarian (Armengol et al., 2007), breast (Rojo et al., 2007), and melanoma (O'Reilly et al., 2009), are associated with poor prognosis and survival. Thus, 4E-BPs may serve as a cell proliferation "brake" in response to a deregulated p53 pathway. p53 is also a regulator of metabolic stress (Murray-Zmijewski et al., 2008), raising the possibility that p53 regulation by 4E-BPs represents a homeostatic mechanism linking translation initiation to the p53 pathway. Such a role for 4E-BPs in this context is supported by studies in *Drosophila* (Teleman et al., 2005; Tettweiler et al., 2005).

Taken together, our findings indicate that a combination of p53 loss and heightened eIF4E activity (due to 4E-BP inactivation) are synergistic in enhancing cell proliferation and tumorigenesis. Although other oncogenic pathways may become engaged upon a reduction in 4E-BP amounts, here we present genetic evidence of how translation initiation and p53 converge in tumorigenesis.

EXPERIMENTAL PROCEDURES

Generation of *4E-BP1* / *4E-BP2* / *p53* / Triple Knockout Mice and Injection of Nude Mice

4E BP1 / *4E BP2* / DKO mice were previously described (Colina et al., 2008; Le Bacquer et al., 2007). *p53* / mice were obtained from Taconic (P53N12 M homozygotes). DKO and *p53* / mice were intercrossed for *4E BP1* / *4E BP2* / *p53* / TKO mice generation. All mice were in the C57BL/6 background. CD1 male nude mice (6 8 weeks old; Charles River Laboratories) were injected subcutaneously with 1 × 10^6 cells in 100 μl phos phate buffered saline. Animal protocols were approved by the McGill Univer sity Animal Care Committee and in compliance to McGill University guidelines.

Cell Lines, Preparation of Primary MEFs, Focus Formation, Soft Agar Assays, and Cell Doubling Assays

MEFs were prepared as described (Le Bacquer et al., 2007). Primary MEFs (between passages 2 and 8) were used. Early passage MRC5 fibroblasts were obtained from American Type Culture Collection. Focus formation and soft agar assays were performed as described (Lazaris Karatzas et al., 1990). For cell doubling assay, MEFs were seeded at 6 × 10^5 cells/100 mm dish and replated every 3 to 5 days. Cell numbers were determined by trypan blue exclusion assays.

Polysomal RNA Preparation and Quantitative Polymerase Chain Reaction

Polysomes were fractionated on sucrose density gradients and RNA was extracted as described elsewhere (Mamane et al., 2007). Quantitative poly merase chain reaction (qPCR) was performed as described previously (Marti neau et al., 2008), using a Mastercycler ep realplex (Eppendorf) or CFX96 (Bio Rad). Quantification was performed using a standard curve derived from serial dilutions of cDNA prepared from WT MEFs. Cycling conditions and primer sequences are provided in Supplemental Data.

Plasmid Construction, Virus Production, Cell Sorting

4E BP cDNAs were subcloned into the BamH1/XhoI sites of the pMSCV IRES GFP plasmid (gift from Jerry Pelletier). pLPC (empty control plasmid), pLPC E1A, pLPC E1A/Ras, and p53shRNA retroviral vectors were gifts from Scott Lowe. Retrovirus production was conducted as described (Le Bacquer et al., 2007). GFP expressing cells were sorted using the FACS Vantage sorter (BD Bioscience). shRNA lentiviruses were prepared as described with some modifications (Stewart et al., 2003). Details are provided in the Supplemental Experimental Procedures. Vectors targeting murine Gas2, human 4E BP1, and human 4E BP2 were obtained from Sigma: TRCN0000088323 (shGas2#1), TRCN0000088324 (shGas2#2), TRCN0000088327 (shGas2#3), TRCN0000040203 (shBP1), TRCN0000117814 (shBP2).

Antibodies

The following antibodies were used: anti p53 and anti 4E BP1 (Cell Signaling), anti 4E BP2 (Banko et al., 2005), anti Gas2 (Abnova), anti $p19^{ARF}$ (Novus), anti p16 (Santa Cruz), anti β actin and anti α tubulin (Sigma).

Measurement of Calpain and SA-β-Galactosidase Activities

Calpain activity in vitro was measured using the InnoZyme Calpain1/2 Activity Assay Kit (Calbiochem). Calpain activity in vivo was measured by flow cytom etry with the substrate t BOC LM CMAC (t butoxycarbonyl Leu Met chloro methylaminocoumarin) (Invitrogen), as described elsewhere (Niapour and Berger, 2007). Cells were fixed and assayed for SA β gal activity as described elsewhere (Dimri et al., 1995).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and seven figures and can be found with this article online at http://www.cell.com/cancer cell/supplemental/S1535 6108(09)00336 5.

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